

# Mitochondrial Nucleotide Variability in Invasive Populations of the Root Weevil *Diaprepes abbreviatus* (Coleoptera: Curculionidae) of Florida and Preliminary Assessment of *Diaprepes* sp. from Dominica

MARINA S. ASCUNCE,<sup>1,2,3</sup> JOEL A. ERNST,<sup>2</sup> ANNEMARIE CLARK,<sup>2</sup> AND HERBERT N. NIGG<sup>1,4</sup>

J. Econ. Entomol. 101(4): 1443–1454 (2008)

**ABSTRACT** *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae) is a root weevil introduced into the United States from the Caribbean in 1964. It is associated with >300 plants, including citrus, sugarcane, and potatoes. *D. abbreviatus* is widespread in Florida, and it has recently been detected in limited areas of California and Texas. The purpose of this research is to evaluate the utility of 16S ribosomal (16S rRNA) and cytochrome oxidase I (COI) mitochondrial markers for the delineation of genetic populations of *D. abbreviatus* in Florida and for the characterization of patterns of dispersion among these populations. We also assessed these markers as genetic tools for the clarification of taxonomic uncertainties in specimens from Dominica (Lesser Antilles). We analyzed 111 weevils from six Florida populations and six specimens from Dominica. In Florida, we found three haplotypes with only one haplotype in each population. Florida haplotypes differed by one to three nucleotide substitutions, possibly the result of a recent divergence from one source population or three different introductions from closely related populations from the Caribbean. In contrast, specimens from Dominica showed a high genetic variability with three 16S haplotypes and six unique COI haplotypes, delineating two mitochondrial clades. We show that these mitochondrial markers are useful for phylogeographic studies of *D. abbreviatus*.

**KEY WORDS** *Diaprepes abbreviatus* L., mitochondrial markers, genetic diversity, invasive species, quarantine pest.

The root weevil *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae) is a polyphagous weevil whose larvae feed on roots and may girdle the crown area of the root system, killing the plant (Griffith 1975). Adult weevils notch the edges of leaves and on rare occasions, may feed on fruit. Field populations of *D. abbreviatus* have a reproductive potential of 7,000–11,000 eggs per female glued between leaves in masses of ≈90 eggs (Nigg et al. 2004). This weevil has a wide range of hosts, with >300 plants, in 59 plant families, including citrus, orchids, sugarcane, corn, peanuts, potatoes, sweet potatoes, papaya, guava, mahogany, hibiscus, cotton, and roses (Simpson et al. 1996, Knapp et al. 2000). *D. abbreviatus* is native in the Caribbean, and it was recorded for the first time in the United States in 1964 in a citrus nursery at Apopka, FL (Woodruff 1964). It was thought to have been introduced through an ornamental plant shipment from Puerto

Rico (Woodruff 1968). After this finding, the Apopka area was monitored and no other specimen was found until 1968 when several adults and larvae were collected in the vicinity of Apopka (Woodruff 1968). Although it disperses slowly and locally (Nigg et al. 2001), *D. abbreviatus* has spread in Florida affecting ≈70,000 ha, of which 30,000 are commercial citrus, with resultant losses to the Florida citrus industry exceeding \$70 million annually (Weissling et al. 2004).

In 2000, *D. abbreviatus* was discovered in a citrus grove in the Rio Grande Valley, Texas (Skaria and French 2001), where it is still present (J. French and B. Thomas, personal communication). Since 1974, weevils have been intercepted several times in California in plant shipments, in truck trailers, and cargo holds on aircraft (Grafton-Cardwell et al. 2004). In September 2005, weevils were found at two sites in Orange County on residential ornamental plants, such as hibiscus and pigmy date palm (K. Godfrey and K. Hoffman, personal communication). In 2006, *D. abbreviatus* was collected in San Diego County, La Jolla (in March) and Encinitas (in July), on avocado and lemons (B. Taylor, personal communication). Currently, in California, 159 ha are infested by *Diaprepes* in Los Angeles, Orange, and San Diego counties. *D. abbreviatus* infestations of homeowner landscapes and

<sup>1</sup> University of Florida, Institute of Food and Agricultural Sciences, Citrus Research and Education Center, 700 Experiment Station Rd., Lake Alfred, FL 33850.

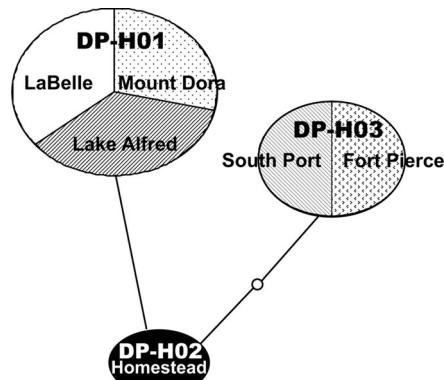
<sup>2</sup> University of Florida, ICBR Genetic Analysis Laboratory, 1376 Mowry Rd., Room 185, Gainesville, FL 32610.

<sup>3</sup> Current address: USDA-ARS Center for Medical, Agricultural and Veterinary Entomology, 1600 SW 23rd Dr., Gainesville, FL 32608.

<sup>4</sup> Corresponding author, e-mail: hnn@ufl.edu.

small private citrus groves represent a threat to citrus and other crops in California.

Because of its wide host range, *D. abbreviatus* poses a threat to other food and ornamental industries in the United States. To prevent the spread of current populations, knowledge about dispersion patterns, sources of current infestations and its mechanism of movement are critical pieces of information to develop effective management strategies. Previous studies analyzed the genetic differentiation of Florida populations of *D. abbreviatus* by esterase polymorphisms and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) markers. Each of these markers showed a different cluster pattern for the populations (Bas et al. 2000). In 1997, the  $\alpha$ -naphthyl-acetate esterase banding pattern analyzed for nine sites in Florida indicated the presence of four clusters (Bas et al. 2000). In 1998, six of these same populations were reanalyzed. In this case, the esterase banding patterns differentiated five of the populations. However, in the 1998 analyses, electrophoresis runs were extended, allowing a better separation of the bands (Bas et al. 2000). Genetic distance measurement based on RAPD banding patterns showed three genetic clusters for these same six populations (Bas et al. 2000). RAPD-PCR data are considered to be more dependable than esterase patterns, as an esterase pattern depends on an active gene(s) (Bas et al. 2000). However, RAPD markers are generated by amplification of random small fragments (200–2,000 based pairs) by using small primers (usually 10 bases), making PCR amplification conditions critical to obtaining reproducibility, the primary shortcoming of RAPDs (Grosberg et al. 1996). The presence or absence of a band at a specific molecular weight is used in the scoring of RAPD alleles. Because of phenotypically dominant expression in diploid organisms, it is generally impossible to distinguish heterozygous from dominant homozygous individuals. In addition to the difficulty of obtaining reproducibility, the underestimation of genetic variation makes the RAPD an unsuitable marker for investigation of migration patterns. Direct sequencing provides a more reliable and repeatable technique. In particular, mitochondrial DNA (mtDNA) sequences are widely used in animal phylogeographic and population genetic studies due in part to the mitochondrial genome (mtDNA) characteristics of maternal inheritance and the absence of recombination (Avise 1994, 2000). Some regions of mtDNA, such as the cytochrome oxidase I (COI) gene, have been extensively used in insects for phylogenetic assessment at varying taxonomic levels (Caterino and Sperling 1999, Normark et al. 1999, Emerson et al. 2000, Cook et al. 2005). Moreover, the mitochondrial COI gene has been chosen as the core of a global bioidentification system for animals by using a portion of the COI sequence as a taxon “barcode” (Hebert et al. 2003, Smith et al. 2006). The 16S ribosomal RNA (rRNA) gene has been combined with COI data to infer phylogenetic relationships and phylogeography in a variety of insects (Pestano et al. 2003, Ribera et al. 2004, Satoh et al. 2004).



**Fig. 1.** Mitochondrial haplotype network for *D. abbreviatus* from Florida based on the combined 16S rRNA and COI sequences. The areas of circles are proportional to the number of samples sharing each haplotype. Lines correspond to single nucleotide mutations and empty circles represent hypothetical haplotypes not observed in the sample.

In this study, we evaluated the utility of mitochondrial markers, 16S rRNA and COI, to identify genetic populations of *D. abbreviatus* in Florida and to characterize patterns of dispersion among these populations. The project in Florida is part of our main research into the genetics of invasive populations of *D. abbreviatus* in the United States (Florida, California, and Texas) and the native populations from the Caribbean islands. Therefore, we included specimens from Dominica to assess of the utility of these markers in the study of the genetic diversity of *Diaprepes* spp. from the Caribbean.

## Materials and Methods

**Sample Collection.** In 2001, 111 weevils were collected by the beat method (Nigg et al. 1999, 2002) from six localities in Florida, five citrus groves (Lake Alfred [ $n = 16$ ], Mount Dora [ $n = 20$ ], LaBelle [ $n = 19$ ], Fort Pierce [ $n = 18$ ], and South Port [ $n = 18$ ]) and one commercial ornamental nursery (Homestead [ $n = 20$ ]) (Fig. 1). In Dominica, six specimens were collected in 2003 from two localities: Castle Bruce (one specimen that was identified as *D. abbreviatus*) and Syndicate ( $n = 5$ , one specimen identified as *D. abbreviatus* and four labeled as *Diaprepes* spp.). Weevils were collected and killed in 70% ethanol, or they were frozen. For overnight shipping, ethanol was decanted. Once in the laboratory, individual specimens were stored in 70–95% ethanol at room temperature. Specimens of this and further genetic studies will be deposited as vouchers at the Florida State Collection of Arthropods, once genetic screening is completed.

**DNA Extraction, PCR Amplification, and Sequencing.** Total DNA was extracted from the hindlimb or head of each individual by using a DNeasy tissue kit (QIAGEN, Valencia, CA) following the manufacturer's protocol for animal tissue. The oligonucleotide primer pairs used for mitochondrial amplification are listed in Table 1. Amplifications were performed in

**Table 1.** Oligonucleotide primers used in this study

| Gene                        | Forward/reverse | Sequence   |
|-----------------------------|-----------------|--|
| 16S rRNA                    | Forward         | A: 5'-CCCTGTTATCAAAACAT-3' <sup>a</sup>                  |
|                             | Reverse         | B: 5'-CTCCGGTTGAAGTCAGATC-3' <sup>a</sup>                |
| <i>Cytochrome oxidase I</i> | Forward         | s1541: 5'-TGAKCYCGAATASTACCAICATC-3' <sup>b</sup>        |
|                             | Reverse         | a2411: 5'-GCTAATCATCTAAAACCTTAATTCCWGTWG-3' <sup>c</sup> |

<sup>a</sup> Kessing et al. (1989).<sup>b</sup> B. Crespi (Simon Fraser University, Burnaby, BC, Canada).<sup>c</sup> Normark et al. (1999).

25- $\mu$ l reactions by using 10 ng of template DNA, 1× PCR buffer, 3 mM MgCl<sub>2</sub>, 0.8 mM dNTP mix, 0.24  $\mu$ M of each primer, and 1 U of *Taq* polymerase (Sigma-Aldrich, St. Louis, MO). Mitochondrial 16S rRNA was amplified using the following parameters: an initial cycle at 94°C (4 min), 35 cycles at 94°C (30 s), 52°C (45 s), 72°C (1 min, 15 s), followed by a final extension at 72°C (5 min). The cytochrome oxidase I gene's PCR conditions included an initial step at 94°C (5 min), 40 cycles at 94°C (30 s), 47°C (60 s, 72°C (60 s), with a final extension of 72°C (10 min). Amplicons were purified using the QIAquick PCR purification kit (QIAGEN) and sent to the ICBR DNA Sequencing Core at the University of Florida for sequencing. Raw data were edited and aligned using Sequencher 3.0 software (Gene Codes Corp., Ann Arbor, MI). Haplotype sequences reported here have been submitted to GenBank (accession nos. EF042119–EF042140).

Nuclear copies of the COI region have been reported in insects (Loxdale and Lushai 1998). Therefore, exploration of potential nuclear transposition of mtDNA fragments (numts) was done, and we found no evidence of numts in our data set. First, all amplifications gave a single and clean PCR product, and no additional peaks were seen in the chromatograms. Second, the COI data that correspond to a protein-coding region were compared with the sequence of *Drosophila yakuba* (Burla) (GenBank accession no. X03240), and their nucleotide sequences were translated into protein sequence by using the software package MEGA version 3.1 (Kumar et al. 2004). The amino acid sequences were checked for the presence of insertions, deletions, and stop codons that would reveal a nonfunctional protein. We found no evidence of a nonfunctional protein.

**Data Analysis.** Mitochondrial sequences were aligned using ClustalX (Thompson et al. 1997). Identical sequences were grouped manually to identify all different haplotypes. The genealogical relationships among haplotypes were analyzed using the program TCS version 1.13 (Clement et al. 2000). A matrix of absolute numbers of pairwise differences among mitochondrial haplotypes was calculated and used to construct a neighbor-joining tree with MEGA version 3.1 (Kumar et al. 2004).

Maximum parsimony analysis was performed using the heuristic search feature of PAUP\* 4.0b10 (Swoford 2003), with 100 random addition replicates. Bootstrapping was performed with 1,000 pseudoreplications of the data set. For the 16S analysis, gaps were treated as a fifth base. Outgroups included the following: *Otiorynchus singularis* (Stephens) (Curculionidae: Entiminae; GenBank accession no. AJ495575) and *Curculio glandium* (Marsham) (Curculionidae: Curculioninae; GenBank accession no. AJ495529) for analysis of the 16S data. For the COI data set, outgroups were *Eurymetopus fallax* (Boheman) (Curculionidae: Entiminae; GenBank accession no. AY790878) and *Sampsonius dampfi* (Schedl) (Curculionidae: Scolytinae; GenBank accession number AF438520).

## Results

**Mitochondrial Variability in *D. abbreviatus* Populations from Florida.** Low genetic diversity, common in invasive species, is present in *D. abbreviatus* populations in Florida. The alignment of 430 bp of the mitochondrial 16S gene in 111 *D. abbreviatus* reveals two haplotypes differing by one transitional substitution (position 254) (Table 2; Appendix 1). In total, 677

**Table 2.** Mitochondrial haplotypes for 16S rRNA (16S), COI (COI), and the concatenated combination (DP) along with collection localities for *D. abbreviatus* from Florida

| Locality    | 16S rRNA |       |       | COI |     |       |       | mtDNA combined |     |     |     |        |        |        |
|-------------|----------|-------|-------|-----|-----|-------|-------|----------------|-----|-----|-----|--------|--------|--------|
|             | 254      | 16S-1 | 16S-2 | 88  | 495 | COI-1 | COI-2 | COI-3          | 254 | 518 | 925 | DP-H01 | DP-H02 | DP-H03 |
| Mount Dora  | C        | 20    |       | T   | T   | 20    |       |                | C   | T   | T   | 20     |        |        |
| Lake Alfred | C        | 16    |       | T   | T   | 16    |       |                | C   | T   | T   | 16     |        |        |
| LaBelle     | C        | 19    |       | T   | T   | 19    |       |                | C   | T   | T   | 19     |        |        |
| Homestead   | C        | 20    |       | T   | C   |       | 20    |                | C   | T   | C   |        | 20     |        |
| Fort Pierce | T        |       | 18    | C   | C   |       |       | 18             | T   | C   | C   |        |        | 18     |
| South Port  | T        |       | 18    | C   | C   |       |       | 18             | T   | C   | C   |        |        | 18     |
| N (111)     |          | 75    | 36    |     |     | 55    | 20    | 36             |     |     |     | 55     | 20     | 36     |

The numbers at the top indicate the locations of variable sites within the corresponding sequence alignments as defined in Supplemental Tables 1 and 2 (16S, 430 bp; COI, 667 bp; and combined, 1,107 bp).



**Fig. 2.** Map depicting the sampling localities for genetic analysis of *D. abbreviatus* in Florida. Localities with circled numbers represent sites sampled in the current study. Localities without circles were sampled for previous publications (Bas et al. 2000).

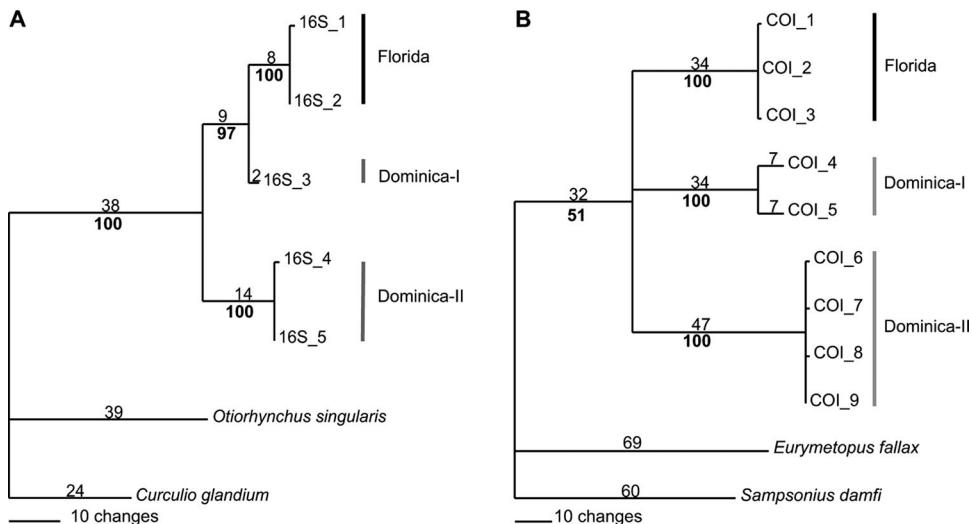
bp of the mitochondrial COI gene analyzed in the same 111 weevils describes three haplotypes, which are separated by one to two transitional substitutions (positions 88 and 495) (Table 2; Appendix 2). Substitutions in the coding gene COI are synonymous and occur at the first position of the triplet (site 88) and at the third position (site 495), respectively. Only one haplotype is present in each population for both markers.

A single alignment of 1107 bp is obtained by combining sequences of both markers of individual weevils. This alignment merges the variable sites observed for each marker, resulting in three variable positions. The haplotype network, constructed based on the 1107-bp alignment, differentiates three haplotypes (Fig. 2). Haplotype DP-H01 is recorded in three localities (Mount Dora, Lake Alfred, and LaBelle) and accounts for 50% of the total sample size (Fig. 2). DP-H01 is connected by one mutational step to DP-H02 (18%), which is observed only in Homestead, and by three mutational steps to DP-H03 (Fig. 2). DP-H03 (32%) is observed in South Port and Fort Pierce, and it is connected to DP-H02 by two mutational steps (Fig. 2).

**Genetic Variability in *Diaprepes* from Dominica.** Contrary to *D. abbreviatus* from Florida, the six *Diaprepes* specimens from Dominica show significant ge-

netic variability accounting for three 16S haplotypes and six unique COI haplotypes. When we pool all the specimens from Dominica, the 430 bp of the 16S alignment present 23 variable sites (Appendix 1). The COI alignment (677 bp) has 107 variable sites, with 14 at the first codon position and 93 at the third codon position (Appendix 2) representing five nonsynonymous substitutions. We also compared the mitochondrial sequences among specimens with consideration of the preliminary taxonomic identification. The two weevils taxonomically identified as *D. abbreviatus* present the same 16S haplotype (16S-3) and two COI haplotypes (COI-4 and -5) differentiated by 14 substitutions at the third codon position with no amino acid change. The four specimens labeled as *Diaprepes* sp. exhibited two 16S haplotypes differentiated by one transition substitution, and four COI haplotypes which present five variable sites, all of which are at third codon positions and synonymous.

**Phylogenetic Relationships in *Diaprepes* Included in This Study.** The bootstrap consensus tree based on the 16S data set supports the monophyly of the *Diaprepes* group, with one basal clade, including two haplotypes from Dominica (Dominica II clade) (Fig. 3A). The other clade groups one haplotype from Dominica and a Florida clade (Fig. 3A). However, the COI bootstrap consensus tree fails to support the mono-



**Fig. 3.** Phylogram of the 50%-majority-rule bootstrap consensus tree based on (A) mitochondrial 16S and (B) COI haplotypes, respectively. Estimated branch lengths ( $>1$ ) are shown above branches, whereas bootstrap values are shown below.

phyly of *Diaprepes* (low bootstrap value), probably due to saturation of the third codon position, but it clearly distinguishes the three mitochondrial lineages observed with the 16S data set (Fig. 3B).

**Measurement of Mitochondrial Differentiation.** We estimated the uncorrected pairwise differences among distinct haplotypes within the three major clades based on the COI data set due to the widespread use of this marker for barcoding in different taxa, which allow further comparisons. Percentage of the uncorrected pairwise differences within clades varied between 0.15 and 2.06%, with Florida haplotypes differing by 0.15–0.30%, Dominica-II clade presented within clade 0.30–0.44% of differences, and Dominica-I reached a within clade differentiation of 2.06% (Table 3). Distance between clades ranged from 10.63 to 10.78% between Florida and Dominica-I, from 12.26 to 12.85% between Florida and Dominica-II, and from 14.47 to 14.92% between Dominica clades.

## Discussion

Accurate identification of pests is essential during interceptions and may provide information of geographic source if a database for comparisons is in place (Armstrong and Ball 2005). Even in species that are already introduced, the ability of fast identification will help to conduct accurate measurements, espe-

cially if geographic origin can be determined. It has been shown that intraspecific hybridization among successively introduced populations may provide the genetic variation necessary for adaptive evolution to occur and thus be a critical determinant of invasive success (Ellstrand and Schierenbeck 2000). Traditional morphological descriptions for the identification of organisms require highly trained personnel, and even this may not be enough to recognize cryptic, unknown species or to define the geographic source of populations. The same concerns apply to early life history stages (eggs and larvae). Molecular diagnostic technology can overcome morphological limitations and provide an accurate and universal identification tool for invasives including geographic source and species identification.

In our study, we evaluated the utility of 16S rRNA and COI mitochondrial markers for the delineation of genetic Florida populations of *D. abbreviatus*, which are characterized by three mitochondrial haplotypes. These molecular tools also were applied in identification of larvae and egg masses found in California, a new area of introduction of *D. abbreviatus*. Among 10 neonates hatched from the same egg mass, we obtained the COI sequences for three of them, which match one of the haplotypes found in Florida. Thirty-one larvae found in California were subject to sequencing for COI, indicating the presence of the same haplotypes found in Florida. In addition, three of these sequences obtained from larvae are identical among themselves and different from *D. abbreviatus*. The blast of these sequences in GenBank shows 95% of identity with the COI sequence of *Asynonychus cervinus* (Curculionidae; also found in Florida) (AY790876). The use of our molecular database provides a tool for monitoring new infestations of *D. abbreviatus* in the United States and can be helpful in

**Table 3.** Ranges of the percentage of uncorrected pairwise differences within (in italics) and among haplotypes based on 677 bp of the COI for major mitochondrial clades within *Diaprepes* included in this study

| Clade       | Florida     | Dominica-I  | Dominica-II |
|-------------|-------------|-------------|-------------|
| Florida     | 0.15–0.30   |             |             |
| Dominica-I  | 10.63–10.78 | 2.06        |             |
| Dominica-II | 12.26–12.85 | 14.47–14.92 | 0.30–0.44   |

**Table 4.** Number of populations of *D. abbreviatus* defined by three different genetic markers in 11 localities from Florida

| Genetic marker<br>Yr of collection | Esterase <sup>a</sup><br>1997 |   |   |   | Esterase <sup>a</sup><br>1998 |   |   |   | RAPDs <sup>b</sup><br>1998 |   |   | mtDNA <sup>c</sup><br>2001 |   |   |   |
|------------------------------------|-------------------------------|---|---|---|-------------------------------|---|---|---|----------------------------|---|---|----------------------------|---|---|---|
|                                    | 1                             | 2 | 3 | 4 | 1                             | 2 | 3 | 4 | 5                          | 1 | 2 | 3                          | 1 | 2 | 3 |
| No. genetic pop                    | 1                             | 2 | 3 | 4 | 1                             | 2 | 3 | 4 | 5                          | 1 | 2 | 3                          | 1 | 2 | 3 |
| Locality                           |                               |   |   |   |                               |   |   |   |                            |   |   |                            |   |   |   |
| Alturas                            | X                             |   |   |   |                               |   |   |   |                            |   |   |                            |   |   |   |
| Fort Pierce                        |                               |   |   |   |                               |   |   |   |                            |   |   |                            |   |   | X |
| Homestead                          |                               |   |   |   | X                             |   |   | X |                            |   |   | X                          |   |   | X |
| LaBelle                            |                               |   |   |   |                               |   |   |   |                            |   |   |                            | X |   |   |
| Lake Alfred                        |                               |   |   |   |                               |   |   |   | X                          |   |   |                            | X |   |   |
| Mount Dora                         |                               | X |   |   |                               | X |   |   |                            | X |   |                            | X |   |   |
| Orlando                            | X                             |   |   |   |                               | X |   |   |                            | X |   |                            |   |   |   |
| Plymouth-Apopka                    | X                             |   |   |   |                               |   |   |   |                            |   |   |                            |   |   |   |
| South Port                         |                               |   |   |   | X                             |   |   |   |                            | X |   |                            | X |   |   |
| Tangerine                          | X                             |   |   |   |                               |   |   |   |                            |   |   |                            |   |   |   |
| Vero Beach (Kerr Center)           |                               |   | X |   |                               |   | X |   |                            |   |   |                            | X |   |   |

The numbers on top indicate the number of the population, and X indicates which localities were clustered as one genetic population.

<sup>a</sup>  $\alpha$ -Naphthylacetate esterase.

<sup>b</sup> Random amplified polymorphic DNA-polymerase chain reaction.

<sup>c</sup> Mitochondrial DNA nucleotide sequences.

control and quarantine decisions (M.S.A., unpublished data).

**Genetic Diversity in *D. abbreviatus* from Florida.** Only one haplotype was found in each of the six sampled populations of *D. abbreviatus* in Florida. Within these six populations, we found three haplotypes differing by one to three substitutions, suggesting that these populations are closely related. It is possible that there is a lack of female migration among the populations with different haplotypes. However, among populations with the same haplotypes, other markers must be evaluated to investigate migration patterns. This possible lack of migration for females seems to be the same for males based on previous nuclear analysis (Bas et al. 2000). *D. abbreviatus* is distributed discontinuously in three quarters of peninsular Florida (central and southern). Mitochondrial haplotypes suggested some phylogeographic pattern with Haplotype 1 and Haplotype 3 on the western and eastern sides of central Florida, respectively, whereas Haplotype 2 was observed in the south. The presence of the same haplotype in geographically distant populations and without a continuous distribution of the weevils, such as in Fort Pierce and South Port, or Mount Dora and LaBelle (Fig. 1), indicates that the most likely mechanism of dispersal in Florida is human transportation of infected plants, as has been suggested previously (Bas et al. 2000). However, studies of the distribution of *Diaprepes* have been conducted mainly on crops (i.e., citrus), whereas the possibility of a more continuous distribution with inclusion of homeowner landscapes needs to be evaluated.

In the absence of genetic data from the natural distribution of the species in the Caribbean, we are unable to make any strong conclusions about the number of invasions. Three potential founder lineages may be represented by haplotypes DP-H01 (Mount Dora-LaBelle-Lake Alfred), DP-H2 (Homestead), and DP-H03 (Fort Pierce-South Port). These may be consistent with three different invasions probably from closely related populations from the Caribbean. How-

ever, fixation of related haplotypes from the same founder population can be an alternative explanation.

Previous nuclear genetic studies of Florida populations of *D. abbreviatus* delineate different numbers of populations depending on techniques and timing (Bas et al. 2000) (Table 4). In particular,  $\alpha$ -naphthylacetate esterase polymorphisms were analyzed in two consecutive years (1997 and 1998), and some populations show nonconcordant genetic patterns (Bas et al. 2000) (Table 4). Considering that biochemical markers are highly sensitive to degradation of the sample and to slight differences in electrophoresis conditions, these features cannot be excluded as the potential source of the differing genetic patterns (Bas et al. 2000). However, the same study shows genetic structure based on RAPD-PCR markers (Bas et al. 2000). RAPD nuclear markers indicate a high level of differentiation among the study populations, whereas genetic distance methods suggest three Florida populations of *D. abbreviatus*: 1) Orlando, 2) Mount Dora, and 3) Lake Alfred-Homestead-South Port-Fort Pierce (Bas et al. 2000) (Table 4; Fig. 4). These three populations are thought to represent three independent introductions of *D. abbreviatus* into Florida (Bas et al. 2000).

These founder populations are different from the populations described by the mitochondrial data; several causes may account for this (Table 4; Fig. 4). Among them is the pattern of inheritance of mitochondrial DNA, which is primarily through females, whereas nuclear DNA inheritance is biparental (Avise 1994). Studies have shown that *D. abbreviatus* disperses slowly and locally (Nigg et al. 2001), and the success of reproduction of migrating females versus migrating males in new populations is undetermined. Moreover, selection may shape differences between populations even when gene flow occurs, and it may affect differently nuclear loci and mitochondrial genes. In *D. abbreviatus* from Florida analyzed by RAPD-PCR, factors such as selection by pesticides and high mutation rates along with local genetic drift cannot be excluded as alternative causes of the differen-



**Fig. 4.** Phenograms of genetic distances based on RAPD allele frequencies on the left and mitochondrial haplotype pairwise differences on the right. Gray and black solid vertical lines are showing the cluster of populations defined by each molecular marker, RAPD and mitochondrial, respectively.

tiation among populations (Bas et al. 2000). These aspects may obscure the demographic history of the original introduction. However, mitochondrial markers (16S and COI) show low variability, which is concordant with the idea that *D. abbreviatus* introduction into Florida is recent. However, to further investigate selection and demographic history and to characterize the pattern of migration of this pest, we need fine-scale resolution neutral markers such as microsatellites. Because microsatellites are noncoding repeated sequences apparently randomly dispersed in the genome, neutrality is expected.

**Genetic Diversity in *Diaprepes* from Dominica.** Further analysis of COI sequences including 31 specimens from Dominica (four sites) indicated the presence of 13 haplotypes (M.S.A., unpublished data). None of these Dominican haplotypes match the haplotypes observed in the United States (168 specimens) nor haplotypes from Puerto Rico ( $n = 42$ ) or the Dominican Republic ( $n = 64$ ) (M.S.A. et al., unpublished data). These data along with the data presented in the current study provide genetic evidence that Dominica may not be the source of *Diaprepes* in Florida. Any invasion of *Diaprepes* species from this or another island of the Caribbean still represents a threat for U.S. agriculture.

Due to uncertainties with initial taxonomic identification of the weevils from Dominica, we consulted Dr. Robert Woodruff, a taxonomic expert on this group. Using the Florida State Collection of Arthropods as a reference, Dr. Woodruff identified all the specimens within the clade Dominica-II as *D. balloui*, a species endemic to Dominica. In this case, morphological identifications are congruent with a molecular clade. Within the Dominica-I clade, he identified the specimen from Castle Bruce as *D. abbreviatus* variety *doublieri* Guerin. He also indicated that the specimen from Syndicate is not *D. abbreviatus*. More specimens should be analyzed to evaluate the species boundaries within the Dominica-I clade.

The sequence differences of 0.1–0.44% observed within Florida and Dominica-II clades are within the ranges described for other species of insects. For example, within *Pissodes schwarzi* (Hopk.) (Coleoptera: Curculionidae) the uncorrected sequence divergence varies between 0.2 and 1.1% (Langor and Sperling 1997) and between 0.2 and 3% in *Baetis rhodani* (Pictet) (Ephemeroptera: Baetidae) (Williams et al. 2006). In *Maoricicada campbelli* (Nyer) (Hemiptera:

Cicadoidea), genetic divergence does not exceed 2.3% (Buckley et al. 2001), whereas higher values such as 3.8% in *Argyrotaenia franciscana* (Walsingham) (Lepidoptera: Tortricidae) are associated with cryptic species (Landry et al. 1999). The Dominica-I clade shows the biggest genetic differentiation at 2.06%, which is close to the species limits described for other insects.

Nucleotide divergence between the Dominica-II and other clades ranged between 12.26 and 14.92%, and values were within the ranges reported for other interspecific comparisons among insects, such as the *Papilio* (L.) species (Lepidoptera: Papilionidae) (Caterino and Sperling 1999). The genetic divergence between the Dominica-I and Florida clades of almost 11% also fits with the interspecific comparisons, and it clearly exceeds values of 5–9.3% observed among *Argyrotaenia* (Stephens) species (Landry et al. 1999). This genetic evidence suggests that the taxonomic status of *D. abbreviatus* variety *doublieri* Guerin needs to be evaluated. Complete morphological phylogenetic analyses of the genus *Diaprepes* need to be conducted, combined with molecular analyses to enhance resolution of species boundaries. The current parsimony analysis, although showing some patterns among the *Diaprepes* sampled, is merely a preliminary attempt at answering these questions.

The highest differentiation (almost 15%) was found between the Dominica clades. Moreover, one specimen from the Dominica-I clade was collected in the same locality as specimens in the Dominica-II clade (Syndicate). The sympatry observed for these two clades can probably be attributed to secondary contact after divergence in allopatry (Category II: major lineage sympatric; Avise 2000; M.S.A. et al., unpublished data).

In conclusion, although the mitochondrial data may help to clarify the phylogeography of *D. abbreviatus* in the United States and to provide a useful tool for early stages identification, a genetic marker that allows fine-scale resolution of the genetic structure of the populations of this pest in Florida would yield valuable information that could be used to 1) identify genetic populations of *D. abbreviatus* in Florida, California, and Texas and to delineate management units; 2) track sources and routes of introduction by comparing U.S. and the Caribbean genetic databases; and 3) describe dispersion patterns of this pest in the United States. A better understanding of these factors will lead to more effective methods of controlling the populations and

limiting the spread of this agricultural pest. We are working to address these issues by using specific polymorphic microsatellites developed for *D. abbreviatus* by Ernst et al. (2006).

### Acknowledgments

We thank Clay McCoy (University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred), Jorge Peña (University of Florida, IFAS, Tropical Research and Education Center, Homestead), Bob Pelosi (University of Florida, IFAS, Indian River Research and Education Center, Fort Pierce), Nadine Cuyler (University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred, collected weevils from Mount Dora), and Harry Anderson (University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred, collected weevils from South Port) for providing weevils from these sites. We are grateful to Bob Woodruff (Florida State Collection of Arthropods, Gainesville) for taxonomic identification of specimens from Dominica and the outline map of Florida from the Florida State Collection of Arthropods. We thank reviewers and editor for comments that helped to improve earlier versions of this manuscript. We thank the ICBR DNA Sequencing Core of the University of Florida for technical support. This research was supported by the Florida Agricultural Experiment Station and a T-STAR grant from the U.S. Department of Agriculture, and the California Department of Food and Agriculture.

### References Cited

- Armstrong, K. F., and S. L. Ball. 2005. DNA barcodes for biosecurity: Invasive species identification. *Phil. Trans. Royal Soc. Biol. Sci.* 360: 1813–1823.
- Avise, J. C. 1994. Molecular Markers, Natural History and Evolution. Chapman and Hall, NY-London.
- Avise, J. C. 2000. Phylogeography: the history and formation of species. Harvard Press, Cambridge, MA.
- Bas, B., Z. Dalkilic, T. L. Peever, H. N. Nigg, S. E. Simpson, F. G. Gmitter, and R. C. Adair. 2000. Genetic relationship among Florida *Diaprepes abbreviatus* (Coleoptera: Curculionidae) populations. *Ann. Entomol. Soc. Am.* 93: 459–467.
- Buckley, T. R., C. Simon, and G. K. Chambers. 2001. Phylogeography of the New Zealand cicada *Mauricicada campbelli* based on mitochondrial DNA sequences: ancient clades associated with Cenozoic environmental change. *Evolution* 55: 1395–1407.
- Caterino, M. S., and F.A.H. Sperling. 1999. *Papilio* phylogeny based on mitochondrial cytochrome oxidase I and II genes. *Mol. Phylogenet. Evol.* 11: 122–137.
- Clement, M., D. Posada, and K. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9: 1657–1660.
- Cook, S., M. Diallo, A. A. Sall, A. Cooper, and E. C. Holmes. 2005. Mitochondrial markers for molecular identification of *Aedes* mosquitoes (Diptera: Culicidae) involved in transmission of arboviral disease in West Africa. *J. Med. Entomol.* 42: 19–28.
- Ellstrand, N. C., and K. A. Schierenbeck. 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? *Proc. Natl. Acad. Sci. U.S.A.* 97: 7043–7050.
- Emerson, B. C., P. Oromí, and G. M. Hewitt. 2000. Tracking colonization and diversification of insect lineages on islands: mitochondrial DNA phylogeography of *Tarphius canariensis* (Coleoptera: Colydiidae) on the Canary Islands. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365: 2199–2205.
- Ernst, J. A., M. S. Ascunce, A. M. Clark, and H. N. Nigg. 2006. Polymorphic microsatellite loci for *Diaprepes* root weevil (*Diaprepes abbreviatus* L.). *Mol. Ecol. Notes* 6: 1–3.
- Grafton-Cardwell, E., K. E. Godfrey, J. E. Peña, C. W. McCoy, and R. F. Luck. 2004. *Diaprepes* root weevil. University of California, Division of Agriculture and Natural Resources, Publication 8131.
- Griffith, R. J. 1975. The West Indian sugarcane root-stalk borer weevil (*Diaprepes abbreviatus*) in Florida. *Proc. Fla. State Hort. Soc.* 88: 87–90.
- Grosberg, R. K., D. Levitan, and B. B. Cameron. 1996. Characterization of genetic structure and genealogies using RAPD-PCR markers: a random primer for the novice and nervous, pp. 67–100. In J. D. Ferraris and S. R. Palumbi [eds.], *Molecular zoology: advances, strategies and protocols*. Wiley, New York.
- Hebert, P.D.N., A. Cywinski, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370: 313–321.
- Kessing, B., H. Croom, A. Martin, C. McIntosh, W. O. McMillan, and S. Palumbi. 1989. The simple fool's guide to PCR. Department of Zoology, University of Hawaii, Honolulu, HI.
- Knapp, J. L., S. E. Simpson, J. E. Peña, and H. N. Nigg. 2000. *Diaprepes* root weevil host list. ENY-641. Department of Entomology and Nematology, University of Florida, Gainesville, FL.
- Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinformatics* 5: 150–163.
- Landry, B., J. A. Powell, and F.A.H. Sperling. 1999. Systematics of the *Argyrotaenia franciscana* (Lepidoptera: Tortricidae) species group: evidence from mitochondrial DNA. *Ann. Entomol. Soc. Am.* 92: 40–46.
- Langor, D. W., and F.A.H. Sperling. 1997. Mitochondrial DNA sequences divergence in weevils of the *Pissodes strobe* species complex (Coleoptera: Curculionidae). *Insect Mol. Biol.* 6: 255–265.
- Loxdale, H. D., and G. Lushai. 1998. Molecular markers in entomology. *Bull. Entomol. Res.* 88: 577–600.
- Nigg, H. N., S. E. Simpson, L. E. Ramos, and N. Cuyler. 1999. Assessment of monitoring techniques for *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae). *Proc. Fla. State Hort. Soc.* 112: 73–77.
- Nigg, H. N., S. E. Simpson, L. E. Ramos, T. Tomerlin, J. M. Harrison, and N. Cuyler. 2001. Distribution and movement of adult *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in a Florida citrus grove. *Fla. Entomol.* 84: 641–651.
- Nigg, H. N., S. E. Simpson, D. G. Hall, L. E. Ramos, S. U. Rehman, B. Bas, and N. Cuyler. 2002. Sampling methods as abundance indices for adult *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in citrus. *J. Econ. Entomol.* 95: 856–861.
- Nigg, H. N., S. E. Simpson, R. J. Stuart, L. K. Yang, R. C. Adair, B. Bas, S. U. Rehman, N. Cuyler, and J. I. Barnes. 2004. Reproductive potential of Florida populations of *Diaprepes abbreviatus* (Coleoptera: Curculionidae). *J. Entomol. Sci.* 39: 251–266.
- Normark, B. B., B. H. Jordal, and B. D. Farrel. 1999. Origin of haplodiploid beetle lineage. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 366: 2253–2259.
- Pestano, J., R. P. Brown, N. M. Suárez, and M. Báez. 2003. Diversification of sympatric *Sapromyza* (Diptera: Lauxaniidae) from Madeira: six morphological species but only four mtDNA lineages. *Mol. Phylogenet. Evol.* 27: 422–428.

- Ribera, I., A. N. Nilsson, and A. P. Vogler. 2004. Phylogeny and historical biogeography of Agabinae diving beetles (Coleoptera) inferred from mitochondrial DNA sequences. Mol. Phylogenet. Evol. 30: 545–562.
- Satoh, A., T. Sota, T. Ueda, Y. Enokido, J. C. Paik, and M. Hori. 2004. Evolutionary history of coastal tiger beetles in Japan based on a comparative phylogeography of four species. Mol. Ecol. 13: 3057–3069.
- Simpson, S. E., H. N. Nigg, N. C. Coile, and R. C. Adair. 1996. *Diaprepes abbreviatus* (Coleoptera: Curculionidae): host plant associations. Environ. Entomol. 25: 333–349.
- Skaria, M., and J. V. French. 2001. *Phytophthora* disease of citrus associated with root weevils in Texas. Phytopathology 91: S203.
- Smith, A. M., N. E. Woodley, D. H. Janzen, W. Hallwachs, and P.D.N. Hebert. 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). Proc. Natl. Acad. Sci. U.S.A. 103: 3657–3662.
- Swofford, D. L. 2003. PAUP\* phylogenetic analysis using parsimony (\*and other methods). Version 4. Sinauer, Sunderland, MA.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX Windows Interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876–4882.
- Weissling, T. J., J. E. Peña, R. M. Giblin-Davis, and J. L. Knapp, Jr. 2004. Featured creatures: *Diaprepes abbreviatus* (Linnaeus). University of Florida Publication EENY-24, Gainesville, FL.
- Williams, H. C., S. J. Ormerod, and M. W. Bruford. 2006. Molecular systematics and phylogeography of the cryptic species complex *Baetis rhodani* (Ephemeroptera, Baetidae). Mol. Phylogenet. Evol. 40: 370–382.
- Woodruff, R. E. 1964. A Puerto Rican weevil new to the United States (Coleoptera: Curculionidae). Florida Department of Agriculture, Division of Plant Industries, Entomol. Circ. 30: 1–2.
- Woodruff, R. E. 1968. The present status of a West Indian weevil *Diaprepes abbreviatus* (L.) in Florida (Coleoptera: Curculionidae). Florida Department of Agriculture, Division of Plant Industries, Entomol. Circ. 77: 1–4.

Received 18 June 2007; accepted 6 December 2007.

---

**Appendix 1.** Alignment of the mitochondrial 16S haplotypes in Diaprepes. Nucleotide substitutions are indicated and dots denote nucleotides identical to the top reference sequence. N denotes ambiguous bases and dashes indicate gaps introduced to align the adjacent 16S sequences. The location of variable sites among haplotypes from Florida are indicated by stars and are in bold

|               |              |   |
|---------------|--------------|---|
| 100           | 16S1_Florida | TGGCTCGGGTAACCTGACCGTGC <sup>A</sup> AAAGGTAGCATAATCATPAGTTTTAATGGAAAGCTGGTATGAAAAGGGTCGGATGAGGAATTTACTGTCCTCTGTGTA |
| 16S2_Florida  | .....        | .....   |
| 16S3_Dominica | .....        | .....   |
| 16S4_Dominica | .....        | .....   |
| 16S5_Dominica | .....        | .....   |
| 200           | 16S1_Florida | AATTAAATTAAATTTTAATTAAAGTAAAAAGCTTAATTTTATGGTAGACGAGAAGACCCCTATAGAGTTTTATTTACTTTTATTAGAAATG-ATGTT                   |
| 16S2_Florida  | .....        | .....   |
| 16S3_Dominica | .....        | .....   |
| 16S4_Dominica | .....        | .....   |
| 16S5_Dominica | .....        | .....   |
| *300          | 16S1_Florida | TAGTATTAGT-AATTTTCTCTATAAAATTAAATTGAAATTGGTTGGGTGATTGAAAAC-TTTCCTTTATAATATCATTGATTATGAGTATAT                        |
| 16S2_Florida  | .....        | .....   |
| 16S3_Dominica | .....        | .....   |
| 16S4_Dominica | .....        | .....   |
| 16S5_Dominica | .....        | .....   |
| 400           | 16S1_Florida | GATCCCTTTTTT-AGATTAAGAAGTAAATTACCTTAAGGATAAACCTGAAATCTTTAAGAGTCAATTCGAAAAGGGGTGCGGACCTCGATCT                        |
| 16S2_Florida  | .....        | .....   |
| 16S3_Dominica | .....        | .....   |
| 16S4_Dominica | .....        | .....   |
| 16S5_Dominica | .....        | .....   |
| 434           | 16S1_Florida | TGGATTAATTTATTATTGCTGTTAGGAGCTAA  |
| 16S2_Florida  | .....        | .....   |
| 16S3_Dominica | .....        | .....   |
| 16S4_Dominica | .....        | .....   |
| 16S5_Dominica | .....        | .....   |

**Appendix 2.** Alignment of the mitochondrial COI haplotypes in Diaprepes. Nucleotide substitutions are indicated and dots denote nucleotides identical to the top reference sequence. N denotes ambiguous bases. The location of variable sites among haplotypes from Florida are indicated by stars and are in bold

## Appendix 2. Continued